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(54) Title: HIGHLY-EFFICIENT, SELF-INACTIVATING, RECOMBINATION-FREE, U3-FREE RETROVIRAL VECTORS

(57) Abstract

The present invention pertains to recombinant retrovirus vectors having a U3-free 5' LTR, a partially deleted 3' LTR, all essential cis-acting sequences for replication, an internal promoter recognizable by a selected host cell, and a non-retroviral gene under the control of the recognized promoter wherein (a) the U3-free 5' LTR is positioned 5' of the non-retroviral gene and has a transcriptional promoter and enhancer different from that of the original retroviral promoter and enhancer replacing the original U3 region of the 5'LTR; (b) the partially deleted 3' LTR is positioned 3' of the non-retroviral gene and has no U3 sequences except for those required at the attachment site for viral integration; (c) an exogenous polyadenylation addition signal sequence recognized by the selected host cell and positioned on the vector 3' to the 3'LTR viral integration site; and (d) the internal recognized promoter is positioned adjacent to the non-retroviral gene on the vector to permit expression of the non-retroviral gene in the host cell; whereby the vector can produce progeny virus in a helper cell with the progeny virus being capable of infecting the selected host cell and forming a provirus in the host cell, with the non-retroviral gene being expressible in the host cell, but the provirus in the host cell will be replication incompetent even in the presence of a helper virus.

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HIGHLY-EFFICIENT, SELF-INACTIVATING, RECOMBINATION-FREE, U3-FREE RETROVIRAL VECTORS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to recombination-free, highly-efficient retroviral vectors that self-inactivate after one round of retroviral replication. The present vectors allow cell-type specific gene expression from tissue specific promoters and enhancers. The recombination-free retroviral vectors eliminate risks of downstream activation of cellular proto-oncogenes (tumorigenesis) that can occur with conventional vectors. This invention also relates to a retrovirus produced by the recombinant retrovirus vector and a host cell containing the provirus of the present invention.

Description of the Background

The disclosures referred to herein to illustrate the background of the invention and to provide additional detail with respect to its practice are incorporated herein by reference. For convenience, the disclosures are referenced in the following text and respectively grouped in the appended bibliography.

Retroviral vectors are the most efficient tools to introduce genes into vertebrate cells. Clinical experiments have been conducted to use retrovirus vectors

to cure a genetic disease in humans (adenosine deaminase (ADA) deficiency). Besides correcting inborn errors of metabolism, gene therapy is also being tested in clinical trials to cure cancer and various other diseases (Science 1992, Vol. 258, pp. 744-746).

Retroviral vectors are basically retroviral particles that contain a crippled viral genome in which all viral protein coding sequences have been replaced with the gene(s) of interest. As a result, such viruses cannot further replicate after one round of infection without the help of a helper virus. Retroviral vector particles are produced by helper cells (Figure 1). Such helper cells contain plasmid constructs which express all retroviral proteins necessary for particle production and replication. After the introduction (transfection) of the retroviral vector genome into such helper cells, the vector genome (an RNA genome) is encapsidated into virus particles (due the presence of specific encapsidation sequences). Virus particles are released from the helper cell carrying a genome containing only the gene(s) of interest (Figure 1). After infection of a fresh target cell, the RNA genome is reverse transcribed into DNA. The DNA copy is integrated into the host genome (Figure 2A). The integrated viral DNA is called the provirus. In the last decade, several retroviral vector systems, derived from chicken or murine retroviruses, have been developed for the expression of various genes (for reviews see Temin, 1987; Gilboa, 1990).

Retroviral vectors have several limitations. For example, one major concern is the possible activation of proto-oncogenes as a result of the integration of the vector into the target cell genome. The activation of proto-oncogenes which is mediated by the viral LTR (Long Terminal Repeats) promoter and enhancer can lead to the malignant transformation (cancer) of the infected cell. Another problem with current retroviral vectors is regulated gene expression. Due to the presence of the retroviral LTR promoter and enhancer. it is impossible to design reliable vectors for tissue specific gene expression.

To avoid these two shortcomings, retroviral vectors have been developed from murine leukemia virus (MLV) and spleen necrosis virus (SNV) that self-inactivate after one round of retroviral replication (Yu et al, 1986; Dougherty and Temin, 1987; United States patent no. 4,980,289 to Temin et al.). This has been achieved by deleting parts or almost all sequences of the retroviral U3 region of the right LTR (Figure 2B). As a result of the mechanism of the retroviral life-cycle, after one round of replication, a retroviral provirus is formed that does not contain LTR promoters.

However, in the case of MLV derived vectors, the efficiency of gene transduction was about four orders of magnitude less than that of a vector with two complete LTRs. In the case of SNV, efficiency of gene transduction decreased only about tenfold. However, detailed investigations revealed that the U3-region of the right LTR was reconstituted in the helper cell (Olson et al., 1992). This reconstitution of the U3-region was the result of recombination (or gene conversion) of the vector with retroviral sequences present in the helper cell or in the other LTR.

To prevent such recombination events, new SNV-derived helper cells were constructed that express retroviral particle proteins from promoters and enhancers different from those present in the retroviral genome. Such new helper cells were termed DSN cells. DSN cells contain two separate plasmids for the expression of gag-pol and env proteins (Dougherty and Temin, 1989). One plasmid contains the gag-pol gene of SNV which is expressed from the CMV promoter. The other plasmid contains the envelope gene unit of SNV which is expressed from the RSV promoter. Experiments performed with these new helper cells and SNV derived U3-minus vectors (e.g., pJD220SVHY, see Figure 3) revealed that the frequency of the recombination event(s) leading to a vector with two wild-type LTRs was markedly reduced. However, vectors containing two wild-type LTRs were still recovered from transfected DSN helper cells (Dougherty et al., 1989). This finding showed that the (unaltered) U3-region of the left LTR of the vector construct can also serve as a template for the recombination (or gene conversion) event resulting in the reconstitution of the U3-region of the right LTR.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a diagram illustrating retroviral helper cells producing recombination-free, self-inactivating retroviral vectors.

FIGURE 2 is a diagram illustrating retroviral vector replication.

FIGURE 3 is a diagram illustrating retroviral vectors derived from spleen necrosis virus (SNV).

FIGURE 4 is a diagram illustrating the nucleotide sequences at the junction of the cytomegalovirus (CMV) with spleen necrosis virus sequences as present in the pPO111 and pPO115 vector series.

SUMMARY OF THE INVENTION

The present invention pertains to a recombinant retrovirus vector having a U3-free 5' LTR, a partially deleted 3' LTR, all essential cis-acting sequences for replication, an internal promoter recognizable by a selected host cell, and a non-retroviral gene under the control of the recognized promoter wherein:

(a) the U3-free 5' LTR is positioned 5' of the non-retroviral gene and has a transcriptional promoter and enhancer different from that of the original retroviral promoter and enhancer replacing the original U3 region of the 5'LTR;

(b) the partially deleted 3' LTR is positioned 3' of the non-retroviral gene and has no U3 sequences except for those required at the attachment site for viral integration;

(c) an exogenous polyadenylation addition signal sequence recognized by the selected host cell and positioned on the vector 3' to the 3' LTR viral integration site; and

(d) the internal recognized promoter is positioned adjacent to the non-retroviral gene on the vector to permit expression of the non-retroviral gene in the host cell;

whereby the vector can produce progeny virus in a helper cell with the progeny virus being capable of infecting the selected host cell and forming a provirus in the host cell, with the non-retroviral gene being expressible in the host cell, but the provirus in the host cell will be replication incompetent even in the presence of a helper virus.

The present invention also pertains to a retrovirus produced by the recombinant retrovirus vector and a host cell containing the provirus of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to recombination-free, highly-efficient retroviral vectors that self-inactivate after one round of retroviral replication. The vectors allow cell-type specific gene expression from tissue specific promoters and enhancers and eliminate risks of downstream activation of cellular proto-oncogenes (tumorigenesis) that can occur with conventional vectors. The present self-inactivating vectors eliminate the problems of recombination and give rise to high gene transfer efficiency, up to 1,000 fold higher than that of existing self-inactivating vectors. The vectors described herein are derived from spleen necrosis virus but vector modification may be made with any retroviral vector leading to similar results. Thus, the novel vectors have a high potential for use in human gene therapy and in other gene transfer applications in which the cell-type specific gene expression is required, e.g., tissue specific gene expression in transgenic animals.

To eliminate vector recombination and improve the efficiency of gene transduction, two modifications were made to a self-inactivating retroviral vector. First, the U3 region of the left LTR was replaced by the immediate early gene promoter and enhancer of the cytomegalovirus (CMV, Figures 3 and 4). This promoter was shown to be the strongest among a series of promoters tested for gene expression in a large variety of cell-types including the SNV promoter in D17 cells. In order not to abolish retroviral replication, the substitution of the U3 promoter by the CMV promoter was done in such a way that initiation of transcription started at the beginning of R as in the wild-type virus. In particular, if RNA transcription is initiated upstream of the original initiation site, first strand cDNA synthesis would result in a cDNA product abolishing replication. It may be transferred and hybridize to the R-region of the second RNA molecule. However, such a cDNA most probably could not function as a primer, since the immediate 3' nucleotides of that cDNA would not find homologous sequences for hybridization. Since the precise initiation of transcription was difficult to predict, three constructs (designated as pPO111-R1, pPO111-R2, and pPO111-R3) were made in which variable amounts of the U3 nucleotides upstream of the first nucleotide of R were retained (Figures 3 and 4). The function as well as frequencies of recombination of these constructs was tested as described earlier (Dornburg and Temin, 1988; Olson et al., 1992) and as described below. Second, the encapsidation region, as present in previous SNV derived vectors, was extended. Such additional sequences increase the efficiency of encapsidation of the vector genome into retroviral particles (Embretson and Temin, 1987).

In one embodiment of the present invention, the vector is a recombinant retrovirus vector having a normally replication incompetent retrovirus gene sequence with a foreign eukaryotic gene. The retrovirus gene sequence is prepared with a deficiency in the retrovirus promoter so that the vector can still produce progeny virus in a helper cell, with the progeny virus being capable of infecting a selected eukaryotic host cell, forming a provirus, and expressing the eukaryotic gene in the host cell, but the provirus will be defective in the retrovirus promoter sequence. Preferably, the vector is a recombinant plasmid. In another embodiment, a foreign internal promoter is positioned adjacent to the foreign eukaryotic gene on the vector to permit expression of the foreign eukaryotic gene in the eukaryotic host cell without initiating retroviral provirus gene expression. In one form, the reading direction of the foreign promoter is inverted relative to the normal reading direction of the retrovirus gene sequence, and a foreign 3' RNA processing sequence is positioned on the side of the foreign eukaryotic gene sequence which is opposite to the foreign promoter. In another embodiment, the retrovirus is a normally replication incompetent retrovirus of the type having a retrovirus portion and a foreign eukaryotic portion, the retrovirus portion having a deficient promoter portion, such that the virus is capable of infecting a eukaryotic host cell, forming a provirus, and expressing a eukaryotic protein coded for by the foreign eukaryotic portion in the host cell, but the provirus will be defective in a retrovirus promoter such that retroviral provirus gene expression doesn't take place in the host cell.

The present invention allows one to select a eukaryotic gene of interest, insert the gene into a vector designed in accordance with the present invention, transfect a helper cell with the vector, harvest virus stock from the helper cell, use the harvested progeny virus to infect a target cell, and have the proviruses which are formed in the target cells express the inserted eukaryotic gene without expressing any retroviral proteins. Since there is no retroviral promoter that is active on the provirus, endogenous helper proteins cannot trigger production of a virus from the provirus. Since there is no retroviral promoter in the provirus, the provirus cannot provide a retrovirus signal that might trigger the host cell to act in an unintended way. The lack of retroviral promoter stops production of retroviral RNA. This system renders much more likely the acceptability of recombinant retrovirus as drugs for vertebrates.

In the present invention, the enhancer and the promoter sequences of the retrovirus present at U3 in SNV have been deleted from the right side of the

DNA sequence in the plasmid vector (3'LTR). Only the sequences required at the attachment site for viral integration in the 3' LTR are present. The U3 5' LTR has a transcriptional promoter and enhancer different from that of the original retroviral promoter and enhancer to replace the original U3 region of the 5'LTR. The total lack of homology in the two U3 sequences prevent recombination. The U3 sequence normally present in the 5' LTR has been reported to be used as a template to repair a partially deleleted U3 sequence in the 3' LTR. By eliminating the U3 sequence in the 5' LTR, the template for repair of the partially deleleted U3 sequence in the 3' LTR is also eliminated. The vector DNA is used to transfect helper cells in a conventional manner. Because transcription from the vector begins at R on the left side, and because the romoter on the left side is not defective, virus can be harvested from the transfected helper cell in the conventional manner. Target cells can then be infected with the harvested virus. Since the right side U3 supplies the coding sequences for both U3 segments in the resulting provirus, the transcriptional promoter which was originally deleted on one side of the plasmid DNA shows up as being deleted from both sides in the resulting provirus. The vector therefore permits a stock of the retrovirus progeny virus to be grown up, yet will not permit further replication after one infection cycle.

If an internal promoter is not inserted, there will be no promoter to produce the desired foreign eukaryotic gene expression. Preferably such a promoter is positioned immediately adjacent to the foreign eukaryotic of interest so that no intervening retrovirus genes RNA is expressed. Because deletion of most of the U3 in SNV resulted in a loss of correct 3' end processing of viral RNA, even though AAUAAA was still present, a polyadenylation site was added to the vector. Moreover, problem recombinations are unlikely because the U3 sequences in the vector are not homologous.

Abbreviations used in the present invention are as follows: pro-promoter; enh-enhancer; PBS-primer binding site for DNA synthesis; PPT-polypurine track for DNA synthesis; E-encapsidation sequences for RNA packaging; attR+-a sequence that will form the right side of the attachment site relating to integration; attL+-the sequence that will form the left side of the attachment side relating to integration; attL+-the deletion of the original provirus left-side attachment site; and attR+-the deletion of the original right side attachment site.

The term "oligonucleotide" as used herein refers to primers, probes, oligomer fragments to be detected, oligomer controls, and unlabeled blocking

oligomers. Oligonucleotide are molecules comprised of two or more deoxyribonucleotides or ribonucleotides. The term "primer" as used herein refers to an oligonucleotide, preferably an oligodeoxyribonucleotide, either naturally occurring such as a purified restriction digest or synthetically produced, which is capable of acting as a point of initiation of synthesis when subjected to conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, *i.e.*, in the presence of nucleotides, an agent for polymerization such as a DNA polymerase, and a suitable temperature and pH. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerization agent.

FIGURE 1 is a diagram illustrating retroviral helper cells producing recombination-free, self-inactivating retroviral vectors. FIGURE 1A shows cells (preferentially D17 cells=canine cells) transfected with two different plasmids expressing the retroviral gag-pol and env genes, respectively. These genes are expressed from promoters and enhancers different from the vector long terminal repeat LTR promoter and enhancer. FIGURE 1B shows that after the transfection of the retroviral vector genome into such helper cells, viral particles are produced that contain the RNA vector genome with the gene of interest.

FIGURE 2 is a diagram illustrating retroviral vector replication. FIGURE 2A shows an example of a conventional retroviral vector provirus as present in the helper cell shown at the top. This vector contains wild-type long terminal repeats with full-length U3, R, and U5 regions. RNA transcription yields the RNA transcript shown below. This RNA contains an encapsidation sequence (E) and all other cis-acting sequences necessary for replication (pbs = primer binding site, ppt = polypurine tract). After infection of a fresh target cell the retroviral genome is reverse transcribed into a double-stranded DNA copy. During this process, the U3-region which contains the retroviral promoter and enhancer and which is present at the 3' end of the RNA genome is duplicated and attached to the 5' end of the DNA copy. In a similar way, the U5 region, present only at the 5' end of the RNA genome, is also duplicated and attached to the 3' end of the DNA copy. The double-stranded DNA copy is integrated into the genome of the infected cell. Due to the presence of two long terminal repeats which contain the retroviral enhancer and promoter, tissue specific gene expression cannot be obtained. Further, the downstream enhancer and promoter can activate gene expression of genes located downstream of the integrated provirus (indicated by an arrow). FIGURE 2B illustrates the principle of a self-inactivating retroviral vector. The vector present in the helper cell is shown at the top. This vector is almost identical

to the one shown above. However, the long terminal repeat at the right side has been modified in the following way: most of U3-sequences including the promoter and enhancer sequences have been deleted. Maintenance of some U3-sequences that form the attachment site (att) is essential for the efficient integration of the vector genome. The RNA transcribed from the U3-minus vector virus does not contain the retroviral promoter and enhancer. Thus, after one round of retroviral replication, a promoter-less retroviral provirus is formed. Genes can be expressed from internal tissue-specific promoters (pro).

FIGURE 3 is a diagram illustrating retroviral vectors derived from spleen necrosis virus (SNV). The SNV genome (provirus) is shown at the top. An example of a standard retroviral derived from SNV is shown below (pJD214HY). In all SNV-derived standard vectors, the protein coding regions (gag-pol and env, the region from SalI to the 3' end of env) have been replaced with the gene(s) of interest (in pJD214HY with hygromycin resistance gene, referred to as hygro). pJD220SVHY is a first generation self-inactivating retroviral vector in which the retroviral promoter and enhancer of the right LTR have been deleted (designated as U3 minus; for more details, see Figure 2B). To increase efficiency of replication, the polyadenylation sequence of simian virus 40 (SV40ter) has been inserted downstream of the U3-minus LTR. To avoid problems of recombination of first generation U3-minus vectors in the pPO111 vectors, the U3 region of the left LTR has been almost completely deleted (except a few nucleotides to guarantee correct initiation of transcription) and substituted for the immediate early promoter and enhancer of the human cytomegalovirus (CMV, for more details, see also Figure 4). Further, this second generation, U3-free vector contains additional retroviral sequences (from SalI to EagI) to increase the efficiency of encapsidation. A universal, second generation U3-free vector (pPO115-R1) contains a multiple cloning site replacing the promoter (SV40pro) and hygromycin B resistance gene (hygro) as present in pJD220SVHY and pPO111-R1 (from EagI to ClaI). The multiple cloning site has been derived from pBluescript II (EagI to ClaI) and allows the easy insertion of various genes and promoters to give new retroviral vectors.

FIGURE 4 is a diagram illustrating the nucleotide sequences at the junction of the cytomegalovirus (CMV) with spleen necrosis virus sequences as present in the pPO111 and pPO115 vector series. An except of the SNV genome showing the TATA box and initiation of transcription is shown at the top. The SacI site downstream of the TATA box of the CMV immediate early promoter (shaded sequence) was used for cloning to connect the CMV promoter and enhancer

to the SNV sequences. pPO111-R2 and pPO111-R3 are 3 or 9 nucleotides shorter than pPO111-R1, respectively.

The function of the new vectors was tested in a tissue culture system as follows and as described earlier (Olson et al., 1992): The new vector constructs pPO111-R1, pPO111-R2, and pPO111-R3 were transfected into helper cells. In parallel, the retroviral vectors pJD214HY, a standard retroviral vector with two complete wild-type LTRs, and pJD220SVHY, the first generation self-inactivating vector from which the new vectors have been derived (Figure 3), were also transfected into helper cell-lines. Transfected cells were selected for hygromycin resistance and cell-lines were established. These resulting helper cell lines are designated as step 1 cells. Virus was harvested from confluent step 1 cultures and fresh helper cells were infected. The infected helper cells were termed step 2 cells. Virus titers were determined (Table 1). We show that the new vectors pPO111-R1 to pPO111-R3 were more efficient than pJD220SVHY. They were almost as efficient as a vector with two wild-type LTRs (pJD214HY).

To test for recombination, virus particles were harvested from confluent step 2 cell cultures (mass infection) and fresh D17 cells were infected. Detection of hygromycin resistant colonies indicates that recombination reconstituted the U3 region: due to the lack of control sequences in RNA transcripts derived from completely U3-minus proviruses, such vectors are not further passaged by retroviral proteins. Thus, only vectors with complete, repaired LTRs are transferred to new target cells. We found that pJD220SVHY was further passaged with high efficiency confirming earlier findings (Olson et al., 1992). The new vectors pPO111-R1 to R3, however, were not further transferred from step 2 helper cells (Table 1). These data show that the new vectors are resistant to recombination events that reconstitute the LTR region.

TABLE 1

EFFICIENCY OF INFECTIVITY AND RECOMBINATION OF SELF-INACTIVATING RETROVIRAL VECTORS DERIVED FROM SNV

	VIRUS TITER (CFU)	
VECTOR NAME	1.INFECTION	2. INFECTION
pJD214HY	6 X 105	5 X 106
pJD220SVHY	8 x 104	1 x 103
pPO111-R1	4 x 105	0*
pPO111-R2	2.5 x 105	0*
pPO111-R1	1.5 x 105	0*

* No hygromycin resistant colonies were obtained with pPO111 vectors.

The retroviral vectors (see also Figure 3) were transfected into helper cells followed by hygromycin selection. Virus was harvested from confluent cultures and fresh helper cells were infected (referred to as first infection). Infected cells were selected for hygromycin resistance, and virus titers were determined (expressed as colony forming units (CFU) per ml of supernatant medium). Cell-lines were established from infected tissue culture plates which contained more than 1,000 independent hygromycin resistant colonies. Virus was harvested from confluent cell cultures and fresh D17 cells were infected (referred to as second infection). Infected cells were selected for hygromycin resistance and virus titers were determined.

Examples

Materials and Methods

Plasmid construction.

The immediate early promoter and enhancer of cytomegalovirus, CMV (a 900 bp Hind3 fragment obtained from plasmid pJDCMV19SV, Dougherty et al., 1990) was cloned into plasmid pRD15 (described in Sheay et al., submitted

for publication). The resulting plasmid was termed pPO101. A DNA fragment containing most of the LTR, the encapsidation region, and parts of the gag-pol region of spleen necrosis virus (a Ball fragment derived from plasmid pSW253, described by Watanabe and Temin, 1982) was cloned into the blunted Sac1 site of pBluescript II KS. The resulting plasmid was termed pPO102. Plasmid pPO102 was digested with Sac1 and re-ligated after treatment with Klenow polymerase 1 (creation of blunt ends) to eliminate a Sac1 site present in the encapsidation sequence. The resulting plasmid was termed (pPO102-S). Using PCR technology and plasmid pPO102-S as template, three DNA fragments were generated comprising slightly different amounts of the SNV R, U5, and encapsidation region: a DNA fragment termed R1 comprises map units 383 to 986 of the SNV genome, fragment R2 comprises map units 385 to 986, and fragment R3 comprises map units 392 to 986. All PCR primers contained Sac1 recognition sites at their 5' ends. After Sac1 digestion, fragments R1, R2, and R3 were cloned into the Sac1 site of pBluescript II KS. The resulting plasmids were termed pPO103-R1, pPO103-R2 and pPO103-R3, respectively. Fragments R1 to R3 were isolated from such plasmids and cloned into the Sac1 site of pPO101. The resulting plasmids were termed pPO104-R1, pPO104-R2, and pPO104-R3, respectively.

Plasmid pJD220SVHY (Dougherty and Temin, 19986) was digested with Ssp1 and Sac1. After Klenow pol1 treatment, a linker coding for the recognition site of the restriction enzyme Bgl2 was ligated. This procedure removed the left LTR promoter, and all of the encapsidation sequences present in this vector. The resulting plasmid was termed pPO106. DNA fragments derived from plasmids pPO104-R1, pPO104-R2, and pPO104-R3 comprising the CMV promoter, R, U5, and the encapsidation sequence (Bgl2 fragments) were isolated and inserted into plasmid pPO106 digested with Bgl2. The resulting plasmids were termed pPO111-R1, pPO111-R2, and pPO111-R3, respectively. The universal retrovirus vectors pPO115-R1 and PPO115-R2 were made by replacing the SV40 promoter and hygromycin B phosphotransferase gene of pPO111-R1 and pPO111-R2 (Eag1 to Cla1 fragment) with the multiple cloning site (Eag1 to Cla1) of pBluescript II KS.

DNA sequence pPO111-R1 5528 b.p. complete sequence;

CAGATCTGAGCTTGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCA
TGTCCAACATTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGG
GTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTG
GCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCA
ATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA
TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGC
ATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATC
GCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACG
GGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGG
ACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGG
GAGGTCTATATAAGCAGAGCTCATCTCTTGCTCGGGGTCGCCGTCCTGCACATTGTTGTTGTGAC
GTGCGGCCCAGATTCGAATCTGTAATAAAACTTTTTTTTTTCTGAATCCTCAGATTGGCAGTGAG
AGGAGATTTTGTTCGTGGTGTTGCCTGGCCTACTGGGTGGGCGCAGGGATCCGGACTGAATCCGT
AGTACTTCGGTACAACATTTGGGGGCTCGTCCGGGATACCCTCCCCATCGGCAGAGGTGCCAACT
GCTTCTTCGAACTTTCTTCGAACTCCGGCGCCGGTGAGTTAAGTACTTGATTTTGGTACCTCGCG
AGGGTTTGGGAGGATCGGAGTGGTGGCGGGACGCTGCCGGGAAGCTCCACCTCCGCTCAGCAGGG
GACGCCCTGACCTGAGCTCGAATTCAGATCTTGTGGTATCTGATTGTTGTTGAGCCGTCCCTAAG
ACGGTGATACTAAGTCGTGGCTTGTGTGTTTGTTTGTTGCCTTGTGTTTGTTCGTCGTTTGTCGA
CAGCGCCTTGCGAATTGGTGTACCCACACCGCGCGGCTTGCGAATAATACTTTGGAGAGCCTTTT
GCCTCCAGTGTCTTCCGTCTGTACTCGTCCTCCTCTCCCTCTCCGGCCGGGAGAGCTCGCCCGGG
GATCCTCTAGTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATAC
ACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGC
GCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCT
GCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGTGGAATGTGTGTCAG
TTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTA
GTCAGCAACCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACC
ATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCC
CCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCC
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GCCCAAGCTGATCCCCGGGGGCAATGAGATATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGA
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DNA sequence pPO111-R2 5525 b.p. complete sequence;

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DNA sequence pPO111-R3 5519 b.p. complete sequence;

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DNA sequence PPO115-R1 3878 b.p. complete sequence;

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CTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG
GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAAT

Throughout this application, various publications have been referenced. The disclosures in these publications are incorporated herein by reference in order to more fully describe the state of the art.

Appendium of References

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W.Sheay, I.Martinez, T.-H.T. Chu. S.Nelson, S.Bhatia, and Ralph Dornburg (1992) The adenovirus leader sequence enhances gene exression when inserted downstream of various promoters in universal gene expression vectors (submitted to "Biotechniques).

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Yu,S.-F., Rueden,T., Kantoff,P.W., Garber,C., Seiberg,M., Ruether,U., Anderson,W.F., Wagner,E.F., and Gilboa,E. 1986. Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proc. Natl. Acad. Sci. 83:3194-3198.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.

I claim:

1. A recombinant retrovirus vector having a U3-free 5' LTR, a partially deleted 3' LTR, all essential cis-acting sequences for replication, an internal promoter recognizable by a selected host cell, and a non-retroviral gene under the control of the recognized promoter wherein:

(a) the U3-free 5' LTR is positioned 5' of the non-retroviral gene and has a transcriptional promoter and enhancer different from that of the original retroviral promoter and enhancer replacing the original U3 region of the 5'LTR;

(b) the partially deleted 3' LTR is positioned 3' of the non-retroviral gene and has no U3 sequences except for those required at the attachment site for viral integration;

(c) an exogenous polyadenylation addition signal sequence recognized by the selected host cell and positioned on the vector 3' to the 3' LTR viral integration site; and

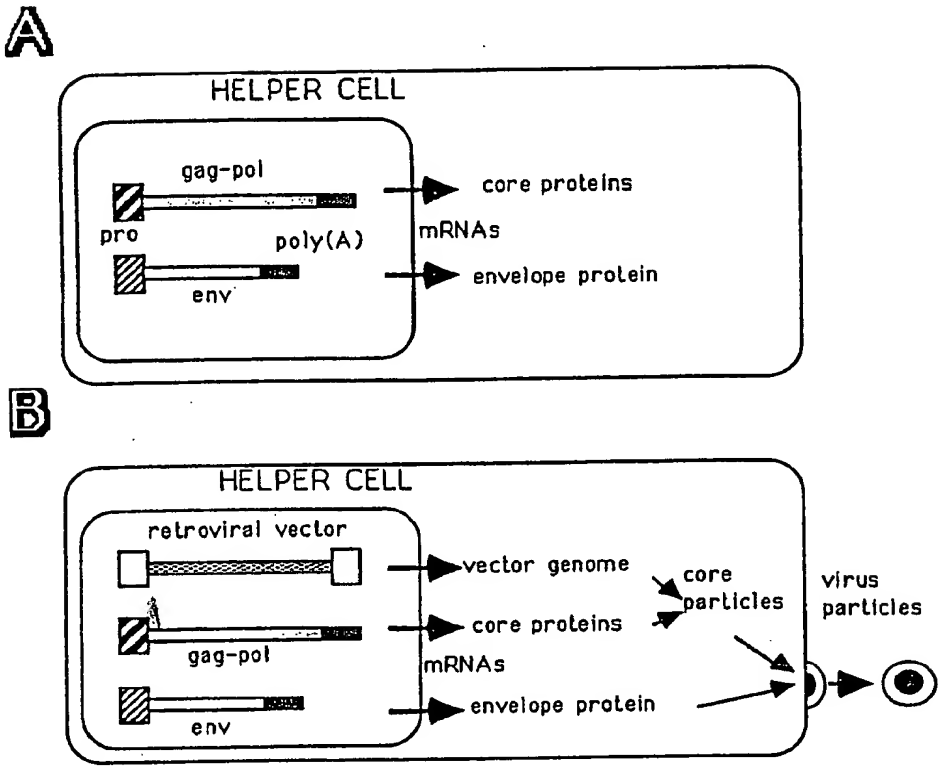
(d) the internal recognized promoter is positioned adjacent to the non-retroviral gene on the vector to permit expression of the non-retroviral gene in the host cell;

whereby the vector can produce progeny virus in a helper cell with the progeny virus being capable of infecting the selected host cell and forming a provirus in the host cell, with the non-retroviral gene being expressible in the host cell, but the provirus in the host cell will be replication incompetent even in the presence of a helper virus.

2. A retrovirus produced by the recombinant retrovirus vector of claim 1.

3. A host cell containing the provirus of claim 1.

FIGURE 1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HELPER CELL
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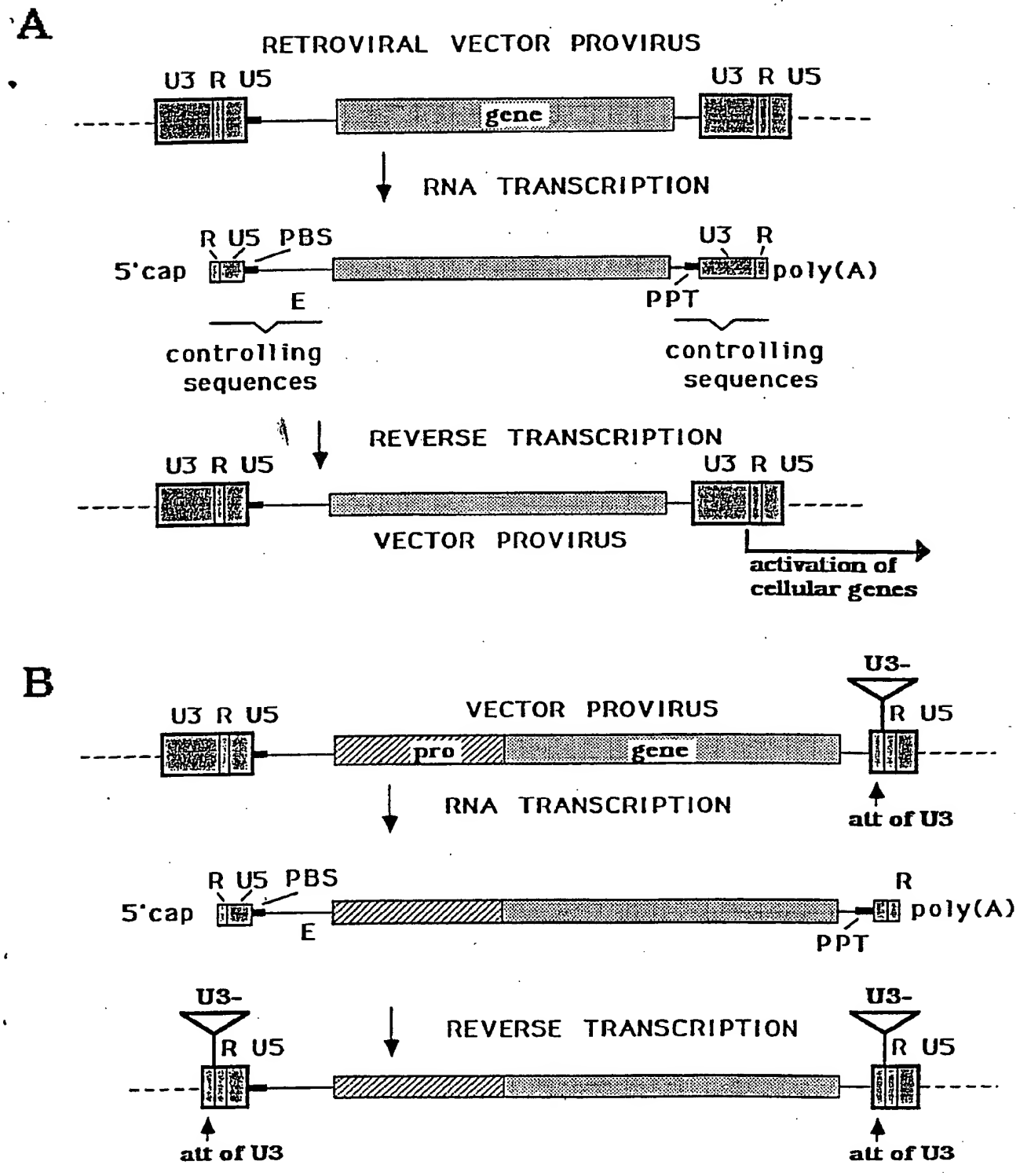
pro
poly(A)
env
core proteins
mRNAs
envelope protein
B
HELPER CELL
retroviral vector
gag-pol
env
vector genome
core proteins
mRNAs
envelope protein
core particles
virus particles

FIGURE 2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gene
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E
PPT
controlling sequences
controlling sequences
REVERSE TRANSCRIPTION
U3 R U5
U3 R U5
VECTOR PROVIRUS
activation of cellular genes
B
U3-
U3 R U5
VECTOR PROVIRUS
R U5
pro
gene
RNA TRANSCRIPTION
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R U5 PBS
R
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R U5
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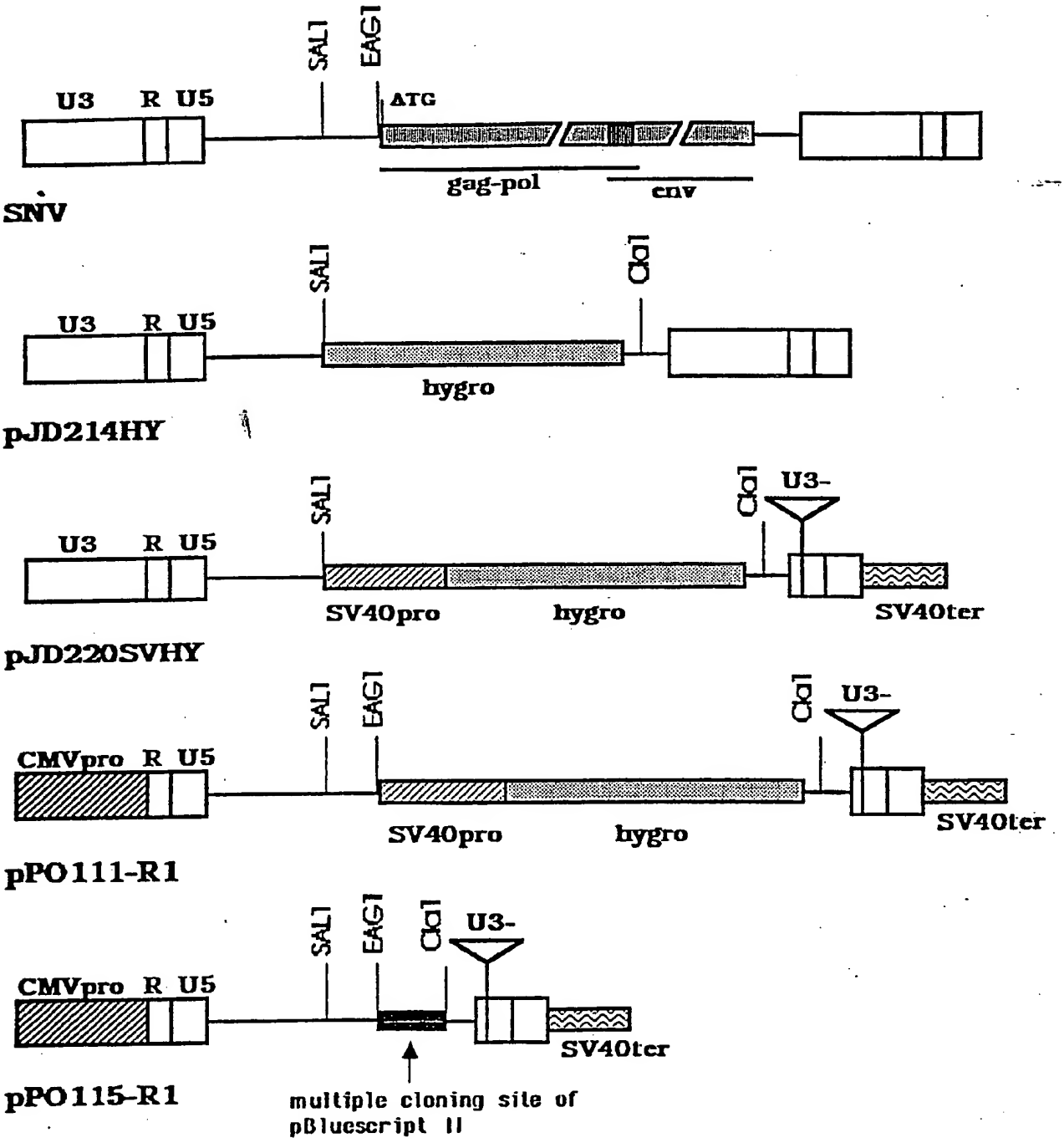
att of U3

FIGURE 3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SAL1
Cla1
U3-
U3
R
U5
SV40pro
hygro
SV40ter
pJD220SVHY
SAL1
EAG1
Cla1
U3-
CMVpro
R
U5
SV40pro
hygro
SV40ter
pPO111-R1
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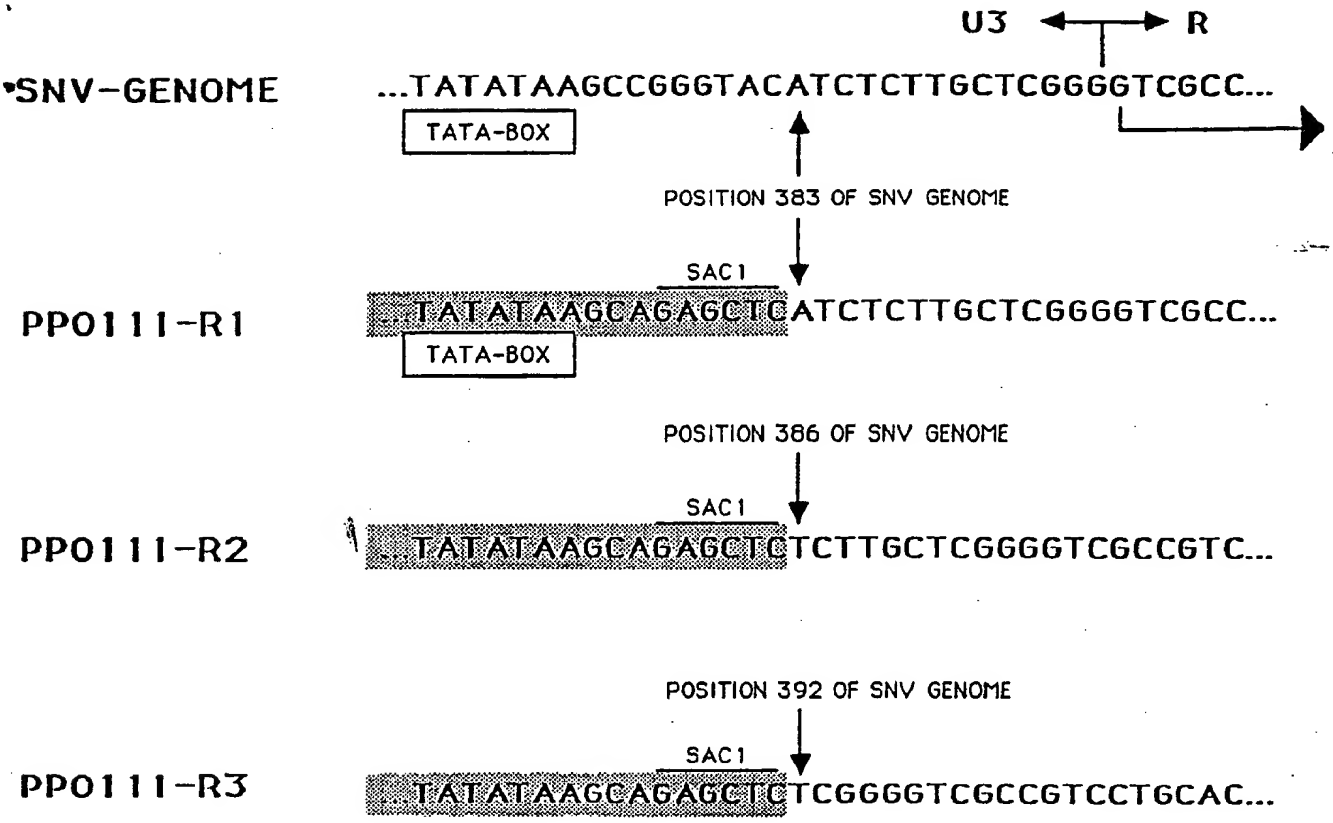
pPO115-R1
multiple cloning site of
pBluescript II

FIGURE 4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TATA-BOX
POSITION 386 OF SNV GENOME
SAC1
PP0111-R2
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06415

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 7/00, 7/01, 15/00, 15/48, 15/85
US CL :435/69.1, 91.1, 91.4, 172.1, 240.2, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 91.1, 91.4, 172.1, 240.2, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Biotech, Medicine, Medline
Search terms: retrovirus, U3, Long terminal repeat, viral vector

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,124,263 (TEMIN ET AL.) 23 June 1992, see whole document.	1-3
Y	Journal of Virology, Volume 66, Number 3, issued March 1992, Olson et al., "Unusually High Frequency of Reconstitution of Long Terminal Repeats in U3-Minus Retrovirus Vectors by DNA Recombination or Gene Conversion", pages 1336-1343, see Figures 1 and 2.	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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